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Shigella flexneri Enters Human Colonic Caco-2 Epithelial Cells through the Basolateral Pole

JOËLLE MOUNIER,[1] THIERRY VASSELON,[1] RAYMOND HELLIO,[2] MAURICE LESOURD,[3] AND PHILIPPE J. SANSONETTI[1]*

Unité de Pathogénie Microbienne Moléculaire et Unité 199, Institut National de la Santé et de la Recherche Médicale,[1] Unité de Biologie des Membranes,[2] and Station Centrale de Microscopie Electronique,[3] Institut Pasteur, 28, Rue du Dr Roux, 75724 Paris Cedex 15, France

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The commonly accepted view that enteroinvasive bacteria enter cells of the intestinal epithelial lining through the apical surface can be challenged in the case of shigellosis. This study is based on in vitro experiments that showed that the invasion of human colonic Caco-2 cells by *Shigella flexneri* occurred through the basolateral pole of these cells. In these experiments, the few bacteria that interacted with the apical surface either bound to microvilli of the cell dome without causing detectable alteration or bound at the level of intercellular junctions at which they demonstrated a limited capacity for paracellular invasion, which permitted subsequent entry through the lateral domain of the cells. Treatment of Caco-2 cell monolayers with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), which disrupts intercellular junctions, greatly enhanced the rate of cell infection. These observations suggest a physiopathological paradox that may have important consequences for the understanding of the process of colonic invasion in vivo during shigellosis.

Shigella flexneri causes bacillary dysentery in humans primarily by invading colonic epithelial cells (19). Therefore, studies of the pathogenesis of this disease have concentrated on the mechanisms by which shigellae invade mammalian epithelial cells grown in vitro (12, 13). These studies have allowed the demonstration and analysis of essential steps of cell invasion, which have been summarized in recent reviews (23, 32). Briefly, in the case of *S. flexneri*, entry into cells occurs via a phagocytic process (7) which is triggered by the products of a set of genes located on a 30-kb sequence (2, 22, 36) of the 220-kb virulence plasmid (34). Following entry, shigellae rapidly lyse the membrane-bound phagocytic vacuole in which they are entrapped, thus gaining free access to the host cell cytoplasm (35). In addition to facilitating rapid intracellular growth (35), access to the cytoplasm allows the invading pathogen to interact with host cell microfilaments, a property by which it induces nucleation and polymerization of actin, thus expressing a capacity to spread intracellularly and from cell to cell (4). In *S. flexneri*, *icsA* (*virG*), another virulence gene located on the 220-kb plasmid, accounts for this intra- and intercellular spread phenotype (Ics) (4, 21, 27). In addition, we have recently described the Olm phenotype (organellelike movement), a second type of intracellular movement in which *S. flexneri* moves within chicken embryo fibroblasts along the cellular stress fibers (42). Olm appears to be an essential mechanism for intracellular colonization of confluent polarized monolayers of Caco-2 cells (43).

Most of these data have been obtained by infecting cells grown to semiconfluency. They cannot be directly extrapolated to the actual invasion process that occurs in vivo because the HeLa and Henle cell lines that are routinely used do not establish confluent polarized monolayers and do not show a clear differentiation of intestinal epithelial cells (26) which have an apical surface that is organized as a brush border and is separated from a basolateral area by well-established intercellular junctions (20).

In an attempt to study the entry process of *S. flexneri* in a cell assay system that reflects more closely the in vivo situation, we used the human colonic epithelial cell line Caco-2, established as a confluent epithelial lining which differentiates into polarized cells that express a well-defined brush border (29). Therefore, the apical surface of these cells was infected under conditions that mimic infection of the human intestinal epithelium.

When this work was initiated, we anticipated two possible outcomes. Either shigellae would enter through the apical surface by disrupting the organization of the microvilli and rearranging the subapical cytoskeleton in order to be phagocytosed, or they would not cross this apical barrier. In the latter case, entry would be likely to occur through the basolateral surface of epithelial cells and would remain limited to a few bacteria unless a significant potential to disrupt intercellular spaces existed.

The use of polarized cells such as MDCK cells has been extremely helpful for the understanding of the invasive mechanisms of *Salmonella choleraesuis* (11). In these cells, as well as in Caco-2 cells (10), salmonellae reaching the apical surface disrupt large areas of the brush-border microvilli and subsequently penetrate by a process resembling phagocytosis. Transepithelial resistance is lost after 3 h of incubation, and severe cellular damage is observed after 6 h. The morphological alterations observed at the level of the cell apex are completely consistent with the pioneering observations made by Takeuchi, who used transmission electron microscopy on sections of the intestine of guinea pigs infected by *Salmonella typhimurium* to demonstrate that entry of the bacteria caused considerable disorganization and blebbing of the enterocyte microvilli (40).

This work demonstrates that, unlike *Salmonella* species, *S. flexneri* does not penetrate through the apical side of polarized Caco-2 cells. The entry process occurs essentially through the basolateral area when it is artificially exposed by disruption of the intercellular junctions, following chelation

* Corresponding author.

of Ca^{2+} contained in the medium (16). *S. flexneri* expresses a limited capacity to disrupt intercellular junctions. It is therefore poorly invasive in this cell assay system. Complex means of invasion probably occur in vivo in order for this pathogen to reach the basolateral area of colonic epithelial cells and subsequently penetrate.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. flexneri* serotype 5 (M90T) was routinely grown in Trypticase soy broth (Diagnostics Pasteur, Marnes la Coquette, France) at 37°C with aeration. Exponentially growing cultures were washed once in phosphate-buffered saline (PBS) and suspended in minimum essential medium (GIBCO Laboratories, Inc., Grand Island, N.Y.) prior to infection of Caco-2 cells.

Culture and infection of Caco-2 cells on plastic dishes. The human colon carcinoma cell line Caco-2 was utilized between passages 69 and 75 (31). Cells were cultured in minimum essential medium with 20% fetal calf serum (GIBCO), which was supplemented with 1% nonessential amino acids (Flow Laboratories, Inc., McLean, Va.), in a 10% CO_2 atmosphere.

Infection of Caco-2 cells with *S. flexneri* was carried out essentially as described previously (25). Briefly, cells were seeded at a concentration of 2×10^5 cells per ml in culture medium on glass coverslips in 35-mm plastic tissue culture dishes. Semiconfluent monolayers were obtained after 48 h of incubation, and confluent monolayers were obtained after 4 days of incubation. Bacteria were added in minimum essential medium at a multiplicity of infection of 100. After centrifugation at 2,000 × *g* for 15 min, plates were incubated at 37°C for various periods.

Culture and infection of Caco-2 cells on filters. Confluent monolayers of Caco-2 cells were obtained in 6 days on Transwell transparent collagen-cellulose filters (Costar Corp., Cambridge, Mass.). The filter diameter was 6.5 mm, and the pore size was 0.4 μm. Cells were then infected as described above, and subsequent enumeration of intracellular bacteria was performed as described below.

Treatment of Caco-2 cells grown on plastic dishes or cellulose filter with EGTA. A confluent, noninfected monolayer was treated with 100 μM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (Sigma Chemical Co., St. Louis, Mo.) in Ca^{2+}-free Krebs-Ringer solution, pH 7.4 (30), for 1 h prior to infection and throughout the incubation period.

Double-fluorescence labeling of bacteria and of the transferrin receptor. Cells were washed once with PBS and once with PHEM {10 mM EGTA, 1 mM $MgCl_2$, 60 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 23 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)}, pH 6.9 (18), and were fixed with 3.7% paraformaldehyde in PBS for 20 min (7). Coverslips were then permeabilized for 1 min in 0.5% Triton X-100 in PHEM. Bacteria were labeled as described previously (7) and revealed by a 1/100 dilution of goat anti-rabbit rhodamine-conjugated immunoglobulin G (Sigma) for 20 min. The receptor for transferrin was stained with a 1/300 dilution of a monoclonal antibody (14) and revealed with a 1/100 dilution of a rabbit anti-mouse fluorescein-conjugated immunoglobulin G (Sigma) for 20 min.

Double-fluorescence labeling of bacteria and of the cell adhesion molecule (L-CAM). After being washed in PBS and PHEM, cells were fixed in acetone at −20°C for 3 min and then permeabilized as described above. Bacteria were labeled by indirect immunofluorescence, and L-CAM was stained with a 1/200 dilution of rabbit polyclonal antiserum (28) and revealed with a 1/100 dilution of goat anti-rabbit fluorescein-conjugated immunoglobulin G (Sigma) for 20 min.

Enumeration of intracellular bacteria. For cells grown on plastic dishes, rough evaluation of intracellular bacteria could be obtained by observing Giemsa-stained preparations at various intervals of time following infection, as illustrated in Fig. 1. On the other hand, quantitative evaluation of the entry process was performed as follows for confluent monolayers established on both plastic dishes and cellulose filters. They were infected in their present condition or after a 1-h treatment with 100 μM EGTA. After centrifugation at 2,000 × *g* for 15 min, followed by incubation for 30 min at 37°C, cells were washed three times with PBS and covered with 2 ml of minimum essential medium without EGTA but with gentamicin (15 μg/ml) for an additional incubation of 1 h in order to kill extracellular bacteria. Cells were then lysed with 0.5% sodium desoxycholate and treated with 5 μg of DNase per ml in 10 mM Tris, pH 7.5, for 30 min at 37°C. Dilutions of the final suspensions were made and plated on Trypticase soy agar. Enumeration of the bacteria on these plates was performed after overnight incubation at 37°C. Data represent the means of six different experiments. Results were then standardized and are given as the number of bacteria per square centimeter of confluent cells.

Confocal microscopy. Fluorescently labeled preparations were observed in a confocal fluorescent imaging system by using a confocal laser scanning microscope (Wild Leitz Instruments GmbH, Heidelberg, Germany). The confocal principle eliminates stray light that does not originate in the focal plane. It therefore optimizes the clarity of images but also allows the demonstration of colocalization of adjacent structures. Confocal sections were generally taken at intervals of 0.4 μm. Pictures were recorded on a flat screen monitor with high linearity.

Scanning electron microscopy (SEM). Cells were grown on 15-mm round plastic tissue culture coverslips (Thermanox; Luc Scientific Corporation, Newberry Park, Calif.). Confluent monolayers were infected as described above. When necessary, EGTA treatment was performed. Cells were then fixed in 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide. Further steps were performed as previously described (1). Samples were observed by using a JEOL JSM-35CF scanning electron microscope.

RESULTS

Infection of confluent Caco-2 cell monolayers by *S. flexneri* on the apical side. Confluent Caco-2 cell monolayers were infected on the apical surface with the invasive strain M90T. After 1, 2, and 4 h of incubation at 37°C, preparations were fixed for Giemsa staining or SEM.

Giemsa-stained preparations showed that only a small number of bacteria interacted with the cells (Fig. 1a). Under these conditions of observation, it was not possible to discriminate between extracellular, intercellular, and intracellular bacteria.

SEM confirmed these results since, with a few exceptions, most of the apical surface of the confluent epithelial monolayers appeared free of interacting bacteria. In rare localized areas, bacteria could be seen interacting with the apical surface. They bound essentially along intercellular junctions, as shown in Fig. 2a. On rare occasions, a few bacteria appeared to bind to microvilli of the enterocyte apical dome, as shown in Fig. 2b, but disruption of these microvilli was

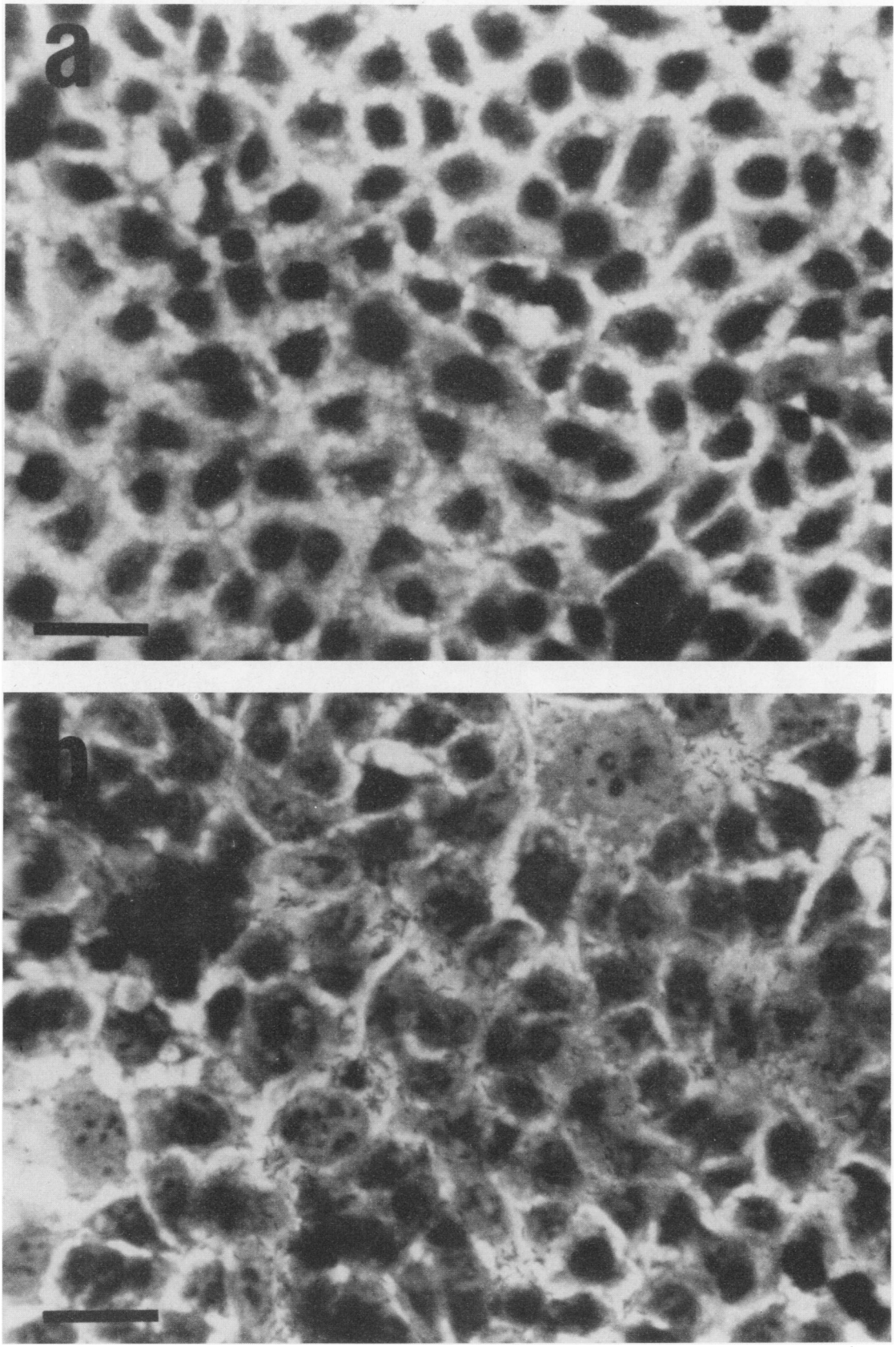


FIG. 1. Giemsa stains of confluent monolayers of Caco-2 cells infected for 2 h with *S. flexneri* M90T. (a) Untreated monolayer; (b) monolayer pretreated with 100 μM EGTA, which disrupts intercellular junctions. Bars = 10 μm.

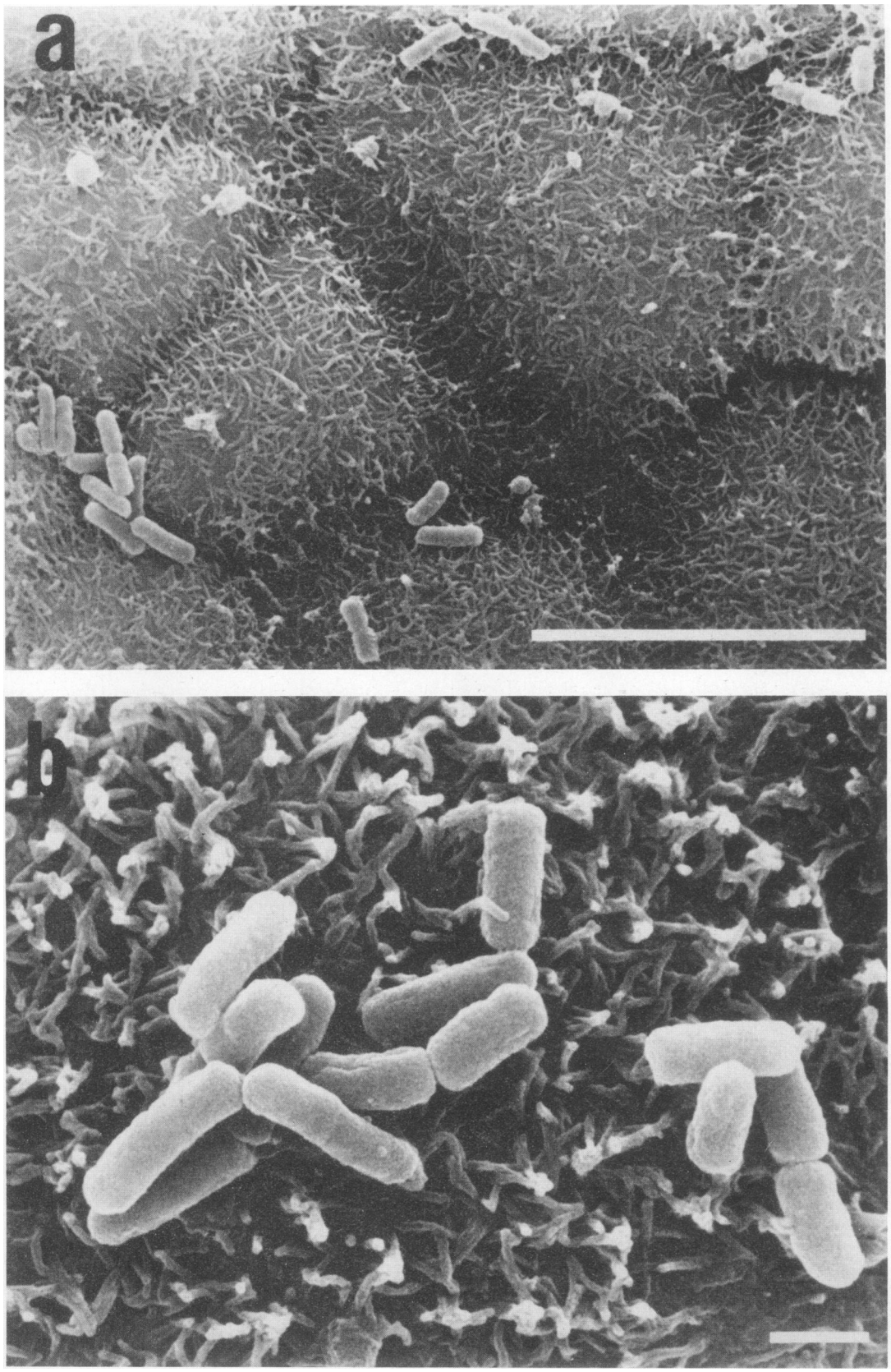


FIG. 2. SEM of a confluent monolayer of Caco-2 cells infected apically for 2 h with *S. flexneri* M90T. (a) Example of an area in which bacteria interacted with the apical surface at the level of intercellular junctions. Bar = 10 μm. (b) Example of one of the few cells on which bacteria bound to the apical dome. Intimate contact was established with no evidence of microvillus disruption and entry. Bar = 1 μm.

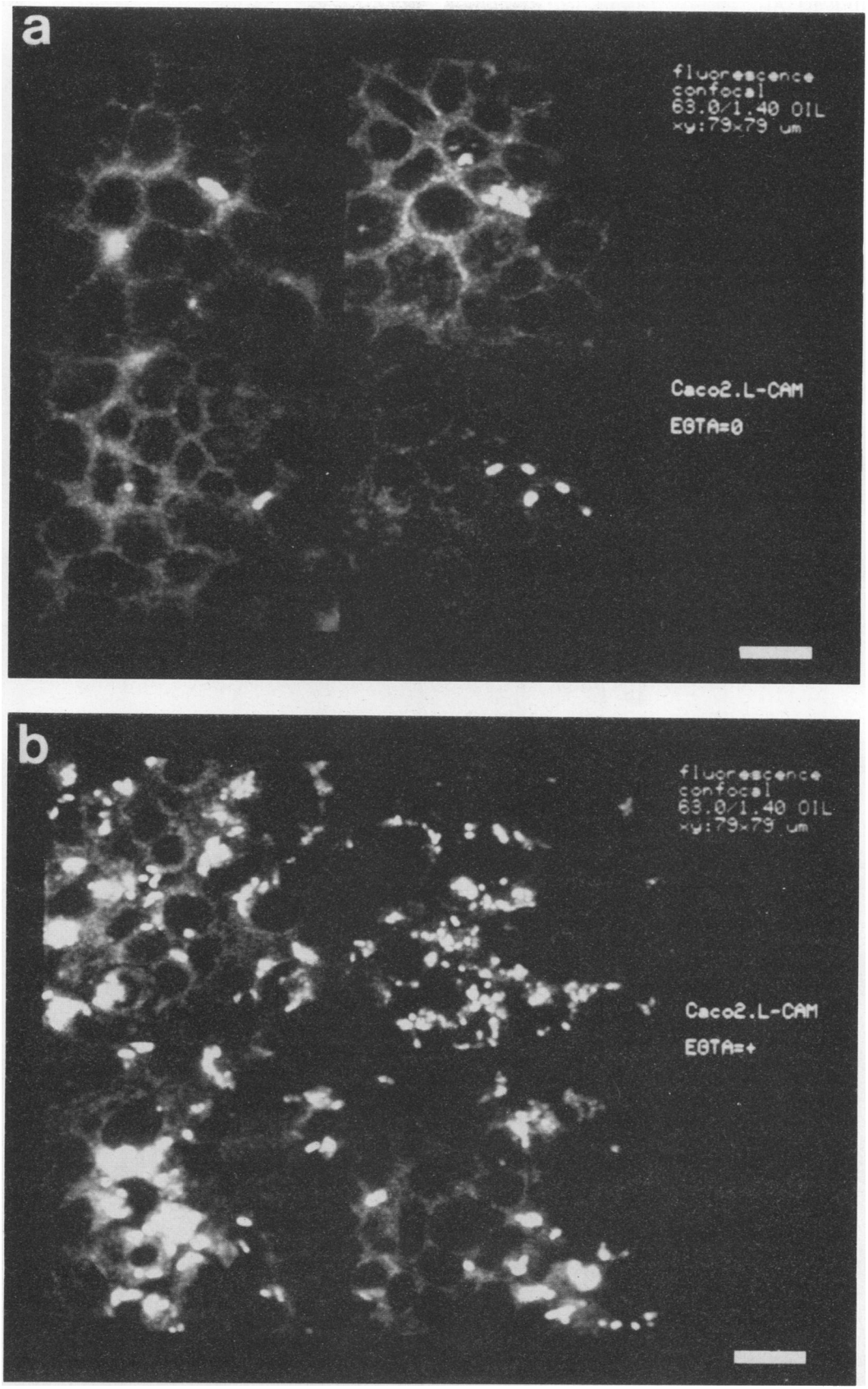
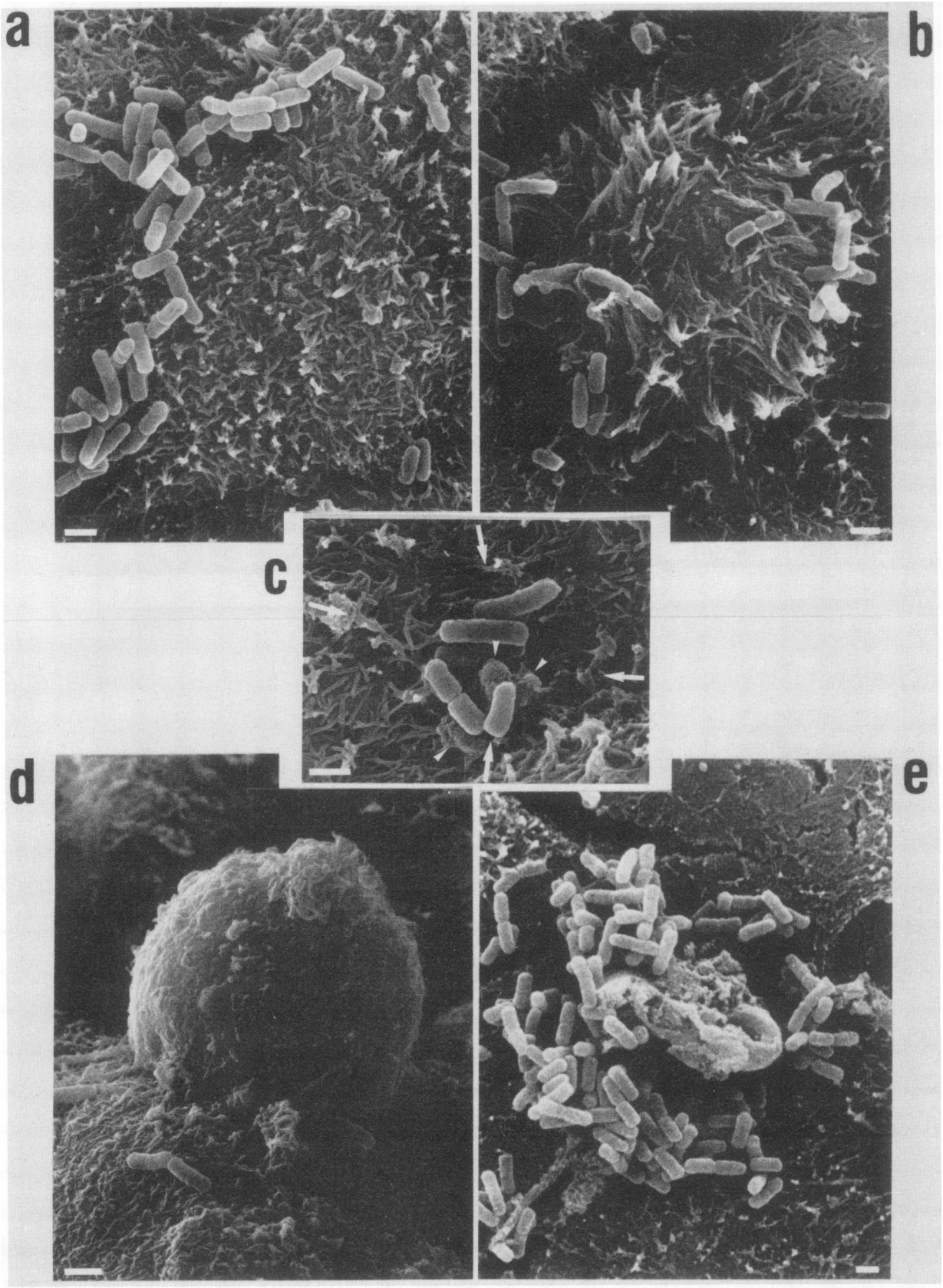


FIG. 3. Confocal microscopic observation of a confluent monolayer of Caco-2 cells infected for 1 h with *S. flexneri* M90T. L-CAM and shigellae were double-fluorescence labeled. Bars = 30 μm. (a) Infection of an untreated monolayer. The plane of focus corresponds to the midportion of the lateral pole of the cells. (b) Infection of a monolayer treated with 100 μM EGTA. Conditions are similar to those for panel a. Both panels a and b contain four different frames corresponding to different areas of the monolayer.


a
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never observed. Moreover, despite scanning of many preparations, we never observed bacteria in the process of penetration of the apical dome. This was consistent with the observation for Giemsa-stained preparations (Fig. 1a) but did not completely rule out the possibility that slight invasion occurred at this level.

In contrast, invasion of the confluent monolayer was observed to occur through the basolateral surface, to a limited degree after 1 h and to a more significant extent after 2 h of incubation. This conclusion is based on two observations. First, a few bacteria were shown to invade the intercellular space and subsequently penetrate into cells from this paracellular location as demonstrated in the following manner. At the end of the incubation period necessary for cell infection, monolayers were carefully washed, permeabilized with Triton X-100, and fixed. Immunofluorescent labeling was performed with both bacteria and L-CAM, a calcium-dependent cell adhesion glycoprotein (17) that is involved both in the formation of specialized epithelial cell junctions and in the establishment of the polarity of these cells (39). Preparations were then observed with a confocal microscope.

Figure 3a shows that, under confocal observation, some of the bacteria that had bound to the epithelial surface had subsequently penetrated the monolayer, since they could be observed in a confocal plane of focus corresponding to the midportion of the cells at the basolateral pole. Bacteria colocalized with L-CAM, thus demonstrating that they had invaded the monolayer through the intercellular space. One of the frames in Fig. 3a shows that some of these bacteria had started to enter adjacent cells from this intercellular compartment.

Second, on rare occasions (i.e., about 1/200 cells), destruction of individual cells, leading to the formation of a gap in the confluent monolayer, could be observed by using SEM. These data are summarized in Fig. 4. A few cells were partially surrounded by a cluster of bacteria binding to the intercellular junction (Fig. 4b). Some of these cells appeared as a rounded dome that began to detach from adjacent cells (Fig. 4d). Ultimately, the dead cell sloughed off and a microcolony of bacteria appeared to invade the monolayer through this opening (Fig. 4e). Figure 4c shows that bacteria had the capacity to alter microvilli in an area restricted to intercellular junctions and cause formation of small pedestals, which are indicated by white arrowheads.

Infection of Caco-2 cell islets by *S. flexneri*. Caco-2 cell islets were cultivated on 35-mm culture dishes. These roughly circular islets contained between 20 and 100 cells which had reached confluency and had displayed proper differentiation marked by the presence of apical microvilli, as observed by using SEM, inside the crown of cells that constitutes the outer edge (data not shown). These islets were infected with M90T and washed after 45 min. Fresh medium containing gentamicin was added in order to kill extracellular bacteria, thereby avoiding further infection, and samples were observed at 1-h intervals. These observations are summarized in Fig. 5. Figure 5a corresponds to a Giemsa-stained preparation that shows that entry of the bacteria occurred only on the outer edge of the cells that limited the islets. Concurrently, only a small number of bacteria were observed interacting with confluent differentiated cells inside the islet at early stages of infection (i.e., 1 h). Bacteria then moved intracellularly and from cell to cell, toward the center of the infected islet, thus allowing complete colonization without passage in the extracellular medium (43).

In an attempt to demonstrate that entry occurred at a specific area of the peripheral cells, expression of the transferrin receptor (14), a specific marker of the basolateral area (37), was performed by using immunolabeling. The outer edge of the cells located at the periphery of the islets expressed this transferrin receptor, thus suggesting that they corresponded to an already differentiated basolateral domain of the cell membrane (Fig. 5b). Double immunofluorescence labeling of invading shigellae and the transferrin receptor demonstrated colocalization (Fig. 5c and d), thus confirming that the site of entry into the peripheral cells of the islet actually corresponded to a basolateral domain of these cells.

This study suggested that shigellae invaded Caco-2 cells through the basolateral pole. This evidence was confirmed in the experiments reported in the next section.

Ca^{2+} depletion of the growth medium enhances *S. flexneri* entry into Caco-2 cells. Cell adhesion molecules such as L-CAM are functionally sensitive to Ca^{2+} concentration (5). Ca^{2+} depletion represents an efficient means of disrupting the cell adhesion apparatus, thus exposing the basolateral pole of the cells. We have exploited this fact in order to allow direct contact of invasive shigellae with this otherwise inaccessible part of the cell surface.

As shown in Fig. 1b, pretreatment of a confluent monolayer with 100 μM EGTA caused a significant increase in the number of shigellae invading Caco-2 cells. The number of bacteria recovered per square centimeter of confluent cells grown on plastic dishes was 1.38×10^4. It increased to 2.83×10^5 after treatment with EGTA, thus indicating a 20-fold increase in the efficiency of infection. Moreover, as shown in Fig. 6, when confluent cells grown on cellulose filters were infected, the number of intracellular bacteria recovered from untreated cells was $9.09 \times 10^2/cm^2$ and reached $2.89 \times 10^5/cm^2$ after treatment with EGTA, thus indicating a 317-fold increase in the efficiency of infection.

In order to confirm that the means by which Ca^{2+} depletion increased entry was by opening intercellular junctions, thus exposing the basolateral pole of the cells, rather than by somehow making the apical pole competent for bacterial entry, double-fluorescence labeling of shigellae and L-CAM, as well as SEM, was performed on monolayers of Caco-2 cells that were infected by strain M90T after treatment with 100 μM EGTA. Following infection, double-immunofluorescence labeling of EGTA-treated Caco-2 cells provided samples that were observed by using confocal microscopy. Compared with data in Fig. 3a, data in Fig. 3b show that a much larger number of bacteria interacted with cells following EGTA treatment. A large majority of the shigellae

FIG. 4. SEM of a confluent monolayer of Caco-2 cells infected for 2 h with *S. flexneri* M90T. This figure shows different steps in the interaction between shigellae and intercellular junctions which lead to cell extrusion and death from the monolayer and invasion of the monolayer through this gap. Bars = 1 μm. (a) Binding of shigellae to intercellular junctions. (b) Rounding of a cell surrounded by bacteria and detachment from neighboring cells. (c) Shigellae localizing at the confluence of four intercellular junctions indicated by white arrows. Microvilli display limited disruption, and the cell membrane appears to form pedestal structures that support the bacteria as indicated by white arrowheads. (d) Extrusion of a cell with bacteria still interacting at the junction level. (e) Extrusion of a dead cell, with a microcolony of shigellae invading the monolayer.

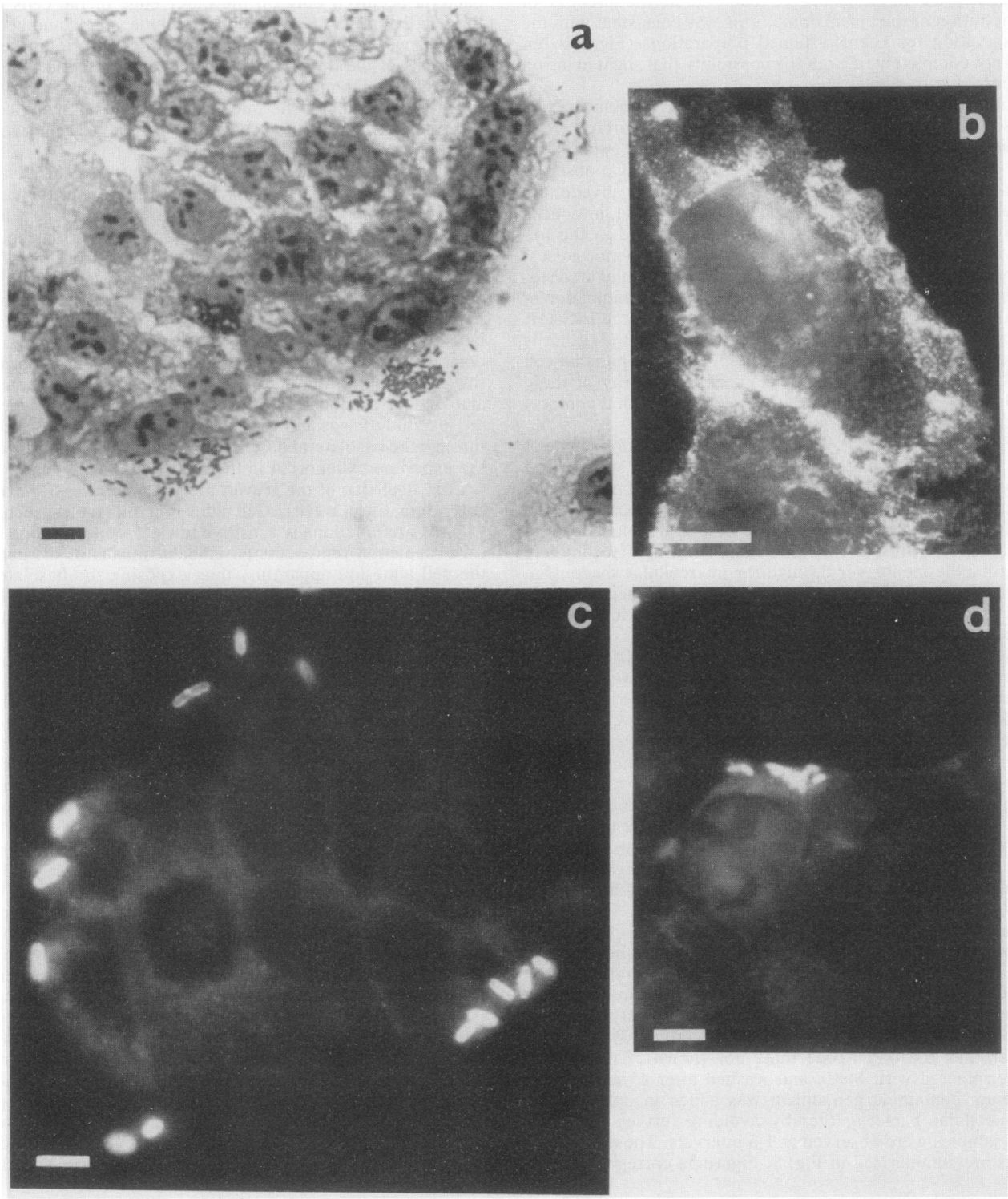

FIG. 5. Infection of Caco-2 cell islets with *S. flexneri* M90T for 1 h. Bars = 10 μm. (a) Giemsa stain which shows the exclusive peripheral location of invasive bacteria. (b) Immunofluorescence labeling of the transferrin receptor, which shows the localization of the receptor on the outer edge of the cells at the periphery of the islet. (c and d) Double immunofluorescence labeling of the transferrin receptor and bacteria. In panel c, labeling of the transferrin receptor on the basolateral area of a Caco-2 cell islet shows a typical "honeycomb" aspect. Bacteria bind to and invade cells only at their peripheral edge, which is labeled by the anti-transferrin antibody as already shown in panel b. Panel d shows that bacterial invasion occurs on cells that express more transferrin receptors, thus indicating that better basolateral differentiation favors entry.

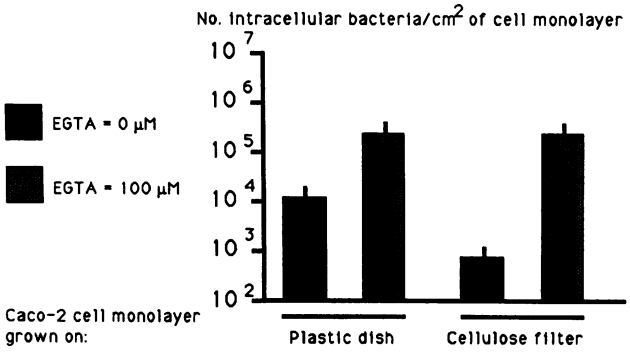

FIG. 6. Rate of infection of confluent Caco-2 cells by strain M90T, which depends on the nature of the support (plastic dishes or cellulose filter) and the presence of EGTA in the medium.

colocalized with L-CAM molecules at the level of the midportion of the cells, thus indicating that they either were located in the intercellular space at the level of the basolateral pole or had started to enter cells from this position.

Observations of similar preparations by using SEM confirmed these data. They are summarized in Fig. 7. Unlike what is seen in Fig. 2, cells appeared clearly separated, exposing the lateral surface totally. The presence of many bacteria, interacting either with cell extensions on the support or with the cell wall itself, was observed in all these intercellular gaps. No larger numbers of bacteria were observed interacting with the apical domes of the cells in this case than in the experiments carried out without EGTA.

DISCUSSION

The critical step in the pathogenesis of shigellosis is invasion of colonic enterocytes by the pathogen (19). It is still unclear, however, how shigellae gain access into these colonocytes. The hypothesis that they penetrate directly through the brush-border microvilli that are characteristic of the apical domain of intestinal cells appears rather simplistic. With the guinea pig intestine, Takeuchi et al. were not able to show evidence of apical penetration by *S. flexneri* (41).

In this work, this hypothesis has been addressed in vitro by studying how *S. flexneri* invades Caco-2 cells grown in tissue culture dishes. In this cell assay system, we have demonstrated that bacteria do not generally enter the monolayer through the apical pole of the cells but rather use the basolateral route, which is not normally exposed. Two lines of evidence support this affirmation.

When cell islets were infected, shigellae entered only through the edge of peripheral cells, in zones identified as basolateral by the expression of the transferrin receptor.

Experiments in which monolayers were treated with EGTA in order to open cellular junctions, thus exposing basolateral areas of the cells, enhanced entry by at least 20 times for cells grown on plastic dishes. This increase most probably represents an underevaluation, since Ca^{2+} depletion of the extracellular medium has been shown to cause a significant decrease in the number of invading bacteria in the HeLa cell invasion assay (8). Moreover, removal of extracellular Ca^{2+} from Madin-Darby bovine kidney epithelial cells induces alterations of membrane-microfilament interactions in the intercellular zonula adherens junctions (44). The occurrence of a similar effect in Caco-2 cells is most likely and would be expected to significantly hamper the phagocytic process. However, when cells grown on cellulose filters were infected under similar conditions, EGTA treatment increased the efficiency of infection by 317-fold, thus indicating that conditions closer to the natural situation, in which cells acquire nutrients from the basolateral area, enhanced the effect of polarity on the rate of bacterial infection.

In the absence of treatment by EGTA, the few bacteria that interacted with the brush border of the apical dome did not alter the structure of the microvilli. These microvilli contain bundles of F-actin cross-linked by villin and bound to the membrane by a complex consisting of a 110-kDa protein belonging to the type 1 myosin family (24) and calmodulin. In order to penetrate this highly organized system via a phagocytic process, the bacterium would be expected to disorganize this pattern, render the membrane-cytoskeleton sufficiently flexible, and mobilize a sufficient pool of actin and actin-binding proteins.

Recognition by the bacterium of an apical surface receptor capable of triggering this cascade of events is a prerequisite that does not at present seem to be fulfilled by *S. flexneri*. On the other hand, in similar cell assay systems, *Salmonella choleraesuis* and *Salmonella typhimurium* cause major disruption of the brush border, which is followed by a phagocytic process (10). The relevant receptor has not yet been identified. The observation that these enteroinvasive microorganisms do not invade epithelial cells by similar routes raises interesting issues regarding the physiopathological consequences of infection.

Among the few bacteria which appeared capable of binding the apical surface of the epithelial lining, most of the microorganisms bound at the level of intercellular junctions. On the basis of this morphological observation, one can suggest that *S. flexneri* recognizes a receptor which is in the region of the zonula occludens. Alternatively, it would be possible that bacteria bind to intercellular junctions in areas in which the zonula adherens is not yet well established or after having destroyed its cellular structure. Recent evidence indicates that I-CAM, a membrane-bound glycoprotein which is an intercellular adhesion molecule, is used as a receptor by certain pathogens such as human rhinoviruses (38) and *Plasmodium falciparum* (3). A molecule such as L-CAM, which would be recognized at the level of the zonula adherens in the absence of a well-established zonula occludens, may also serve as a receptor mediating this binding.

Since early after infection had been initiated the few bacteria able to invade the confluent monolayer appeared to colocalize with L-CAM molecules, they apparently entered the monolayer through the basolateral pole of the cells. This observation implies that bacteria have the capacity to pass through the tight junctions of the cells, thus following a paracellular pathway. Observation of preparations by using SEM suggested two means by which shigellae may reach the intercellular space, which were identified by the presence of L-CAM molecules as well as the expression of the transferrin receptor. Some of the bacteria that bind to the intercellular junctions of the cells on the apical pole may be able to cause local disruption of the junctions and progress further in the intercellular space before reaching the zone of the basolateral pole that mediates the entry process. Recent evidence that microorganisms such as *Vibrio cholerae* (9) and *Clostridium difficile* (15) express toxins that damage the tight junctions suggests that this process could also exist in vivo for *S. flexneri*. In addition, in a few instances, some bacteria were observed to surround a cell by binding to its

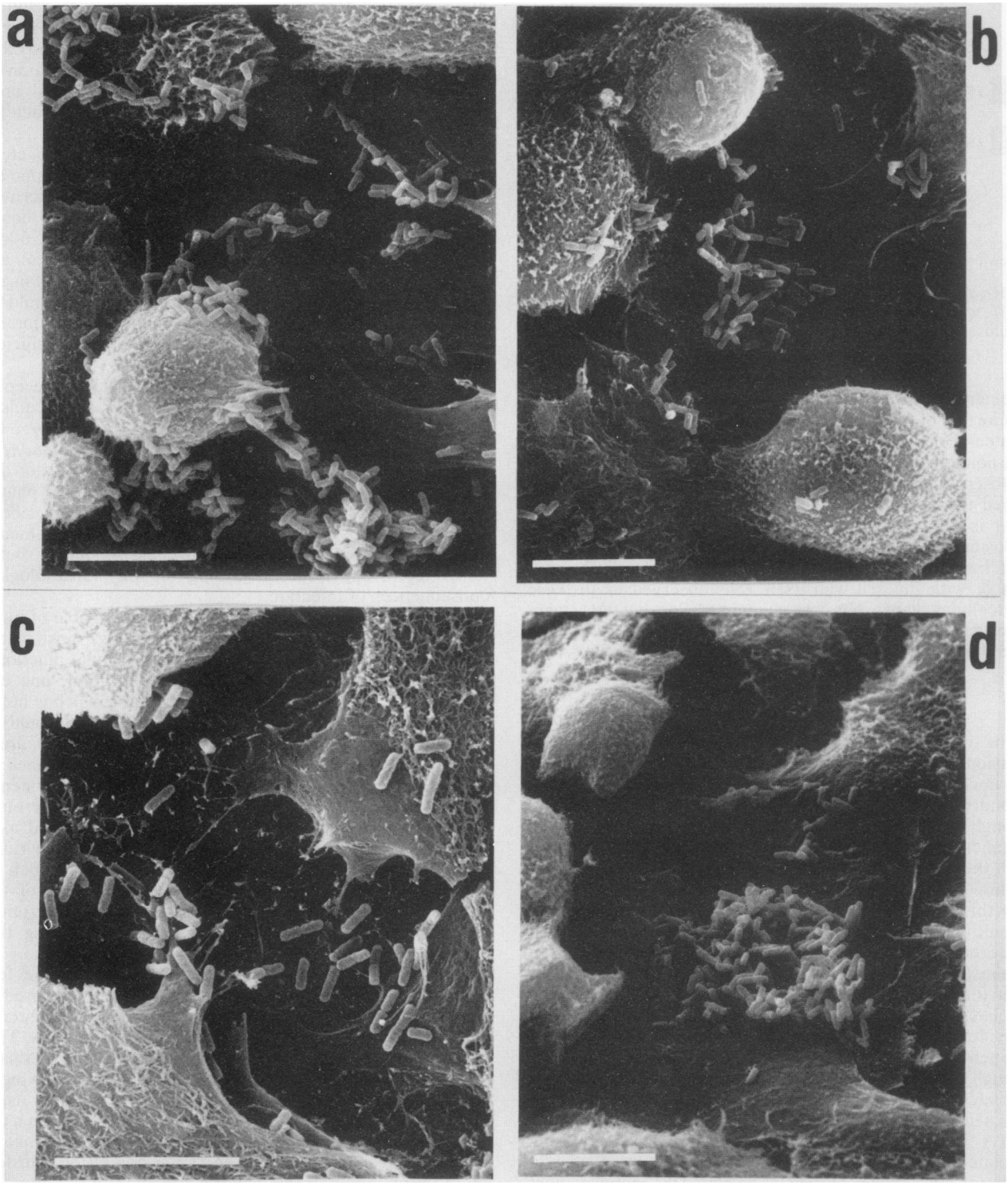

FIG. 7. SEM of a confluent monolayer of Caco-2 cells treated with 100 μM EGTA and infected for 2 h with *S. flexneri* M90T. Panels a to d are characteristic views of shigellae interacting with the cells' basolateral domain exposed following disruption of intercellular junctions. Bars = 10 μm.

intercellular junction. This caused detachment and death of the target cell, thus allowing access of the bacterial microcolony to the basolateral pole. Such a situation may very well exist in vivo, particularly in colonic crypts, in which the clearance of luminal bacteria is limited.

Moreover, as mature enterocytes slough off the tip of colonic villi, transient opening of the epithelial lining may allow invasion by bacteria. We are currently testing these various hypotheses by using cell assay systems as well as more definitive models, such as rabbit ligated ileal loops.

However, we have recently made an interesting observation with macaque monkeys infected intragastrically either by a wild-type isolate of *S. flexneri* or by an *icsA* mutant that does not spread intracellularly or from cell to cell (33). We had anticipated that this mutant would aid in the identification of the initial site of invasion by shigellae of the colonic mucosa. Animals infected with this mutant developed moderate symptoms of dysentery compared with those infected with the wild-type strain. Under colonoscopic examination, minor abscesses and ulcerations could be observed which, upon histopathological examination, appeared to be located over lymphoid follicles. This indicates that shigellae invade the colonic epithelium preferentially in those areas rich in M cells, which are involved in antigen sampling from the intestinal lumen (6). Data obtained by Wassef et al. (45) are in agreement with this hypothesis. M cells may therefore act as a "Trojan horse," allowing shigellae to bypass the apical surface of epithelial cells, which are quite refractory to invasion. From these cells, bacteria may reach the subepithelial tissues and infect colonocytes via the basolateral pole or pass from one cell to another by expressing the Ics phenotype.

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REFERENCES

1. **Arro, E., V. P. Collins, and U. T. Brunk.** 1981. High resolution SEM of cultured cells: preparatory procedures. Scanning Electron Microsc. **II**:159–168.
2. **Baudry, B., A. T. Maurelli, P. Clerc, J. C. Sadoff, and P. J. Sansonetti.** 1987. Localization of plasmid DNA loci necessary for the entry of *Shigella flexneri* into HeLa cells, and characterization of one locus encoding four immunogenic polypeptides. J. Gen. Microbiol. **133**:3409–3413.
3. **Berendt, A. R., D. L. Simmons, J. Tansey, C. I. Newbold, and K. Marsh.** 1989. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature (London) **341**:57–59.
4. **Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti.** 1989. Identification of *icsA*, a plasmid locus of Shigella flexneri that governs bacterial intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA **86**:3867–3871.
5. **Boyer, B., and J. P. Thiery.** 1989. Epithelial cell adhesion mechanisms. J. Membr. Biol. **112**:97–108.
6. **Bye, W. A., C. H. Allan, and J. S. Trier.** 1984. Structure, distribution and origin of M cells in Peyer's patches of mouse ileum. Gastroenterology **86**:789–801.
7. **Clerc, P., and P. J. Sansonetti.** 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55**:2681–2688.
8. **Clerc, P. L., B. Berthon, M. Claret, and P. J. Sansonetti.** 1989. Internalization of *Shigella flexneri* into HeLa cells occurs without an increase in cytosolic Ca^{2+} concentration. Infect. Immun. **57**:2919–2922.
9. **Fasano, A., B. Baudry, D. W. Pumplin, S. S. Wasserman, B. D. Tall, J. M. Ketley, and J. B. Kaper.** *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Natl. Acad. Sci. USA, in press.
10. **Finlay, B. B., and S. Falkow.** 1990. Salmonella interaction with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. **162**:1096–1106.
11. **Finlay, B. B., B. Gumbiner, and S. Falkow.** 1988. Penetration of Salmonella through a Madin-Darby canine kidney epithelial cell monolayer. J. Cell Biol. **107**:221–230.
12. **Hale, T. L., and P. F. Bonventre.** 1979. Shigella infection of Henle intestinal epithelial cells: role of the bacteria. Infect. Immun. **24**:879–886.
13. **Hale, T. L., R. E. Morris, and P. F. Bonventre.** 1979. Shigella infection of Henle intestinal epithelial cells: role of the host cell. Infect. Immun. **24**:887–894.
14. **Haynes, B. F., M. Hemler, T. Cotner, D. L. Mann, G. S. Eisenbarth, J. L. Strominger, and A. L. Fauci.** 1981. Characterization of a monoclonal antibody (5E9) that defines a human cell surface antigen of cell activation. J. Immunol. **127**:347–351.
15. **Hecht, G., C. Pothoulakis, J. T. LaMont, and J. L. Madara.** 1988. Clostridium difficile toxin A perturbs cytoskeletal structures and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. **82**:1516–1524.
16. **Kartenbeck, J., E. Schmid, W. W. Franke, and B. Geiger.** 1982. Different modes of internalization of proteins associated with *adherens* junctions and desmosomes. EMBO J. **1**:725–732.
17. **Kemler, R., C. Babinet, H. Eisen, F. Jacob.** 1977. Surface antigen in early differentiation. Proc. Natl. Acad. Sci. USA **74**:4449–4452.
18. **Kuhn, M., M.-C. Prévost, J. Mounier, and P. J. Sansonetti.** 1990. A nonvirulent mutant of *Listeria monocytogenes* does not move intracellularly but still induces polymerization of actin. Infect. Immun. **58**:3477–3486.
19. **Labrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
20. **Madara, J. L., and G. Hecht.** 1989. Tight (occluding) junctions in cultured (and native) epithelial cells, p. 131–163. *In* K. S. Matlin and J. D. Valentich (ed.), Functional epithelial cells in culture. Alan R. Liss, Inc., New York.
21. **Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa.** A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *Shigella flexneri*. Cell **46**:551–555.
22. **Maurelli, A. T., B. Baudry, H. d'Hauteville, T. L. Hale, and P. J. Sansonetti.** 1985. Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. **49**:164–171.
23. **Maurelli, A. T., and P. J. Sansonetti.** 1988. Genetic determinants of Shigella pathogenicity. Annu. Rev. Microbiol. **42**:127–150.
24. **Mooseker, M. S., and T. R. Coleman.** 1989. The 110-kD protein-calmodulin complex of the intestinal microvillus (brush border myosin I) is a mechanoenzyme. J. Cell Biol. **108**:2395–2400.
25. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. **58**:1048–1058.
26. **Neutra, M., and D. Louvard.** 1989. Differentiation of intestinal cells in vitro, p. 363–398. *In* K. S. Matlin and J. D. Valentich (ed.), Functional epithelial cells in culture. Alan R. Liss, Inc., New York.
27. **Pál, T., J. W. Newland, B. D. Tall, S. B. Formal, and T. L. Hale.** 1989. Intracellular spread of *Shigella flexneri* associated with the *kcpA* locus and a 140-kilodalton protein. Infect. Immun. **57**:477–486.
28. **Peyriéras, N., D. Louvard, and F. Jacob.** 1985. Characterization of antigens recognized by monoclonal and polyclonal antibodies directed against uvomorulin. Proc. Natl. Acad. Sci. USA **82**: 8067–8071.
29. **Pinto, M., S. Robine-Leon, M. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum.** 1983. Enterocyte-like differentiation of the human colon carcinoma cell line, Caco-2 in culture. Biol. Cell **47**:323–330.
30. **Pitelka, D. R., B. N. Taggart, and S. T. Hamamoto.** 1983. Effect of extracellular calcium depletion on membrane topography and occluding junctions of mammary epithelial cells in culture. J. Cell Biol. **96**:613–624.
31. **Rousset, M.** 1986. The human colon carcinoma cell lines HT-29

and Caco-2: two *in vitro* models for the study of intestinal differentiation. Biochimie **68:**1035–1040.

32. **Sansonetti, P. J.** 1991. Genetic and molecular basis of cell invasion by Shigella spp. Rev. Infect. Dis. **13**(Suppl. 4)**:**285–292.
33. **Sansonetti, P. J., J. Arondel, A. Fontaine, H. d'Hauteville, and M. L. Bernardini.** 1991. ompB (osmo-regulation) and icsA (cell to cell spread) mutants of *Shigella flexneri*: vaccine candidates and probes to study the pathogenesis of shigellosis. Vaccine **9:**416–422.
34. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35:**852–860.
35. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51:**461–469.
36. **Sasakawa, C., K. Kamata, T. Sakai, S. Makino, M. Yamada, N. Okada, and M. Yoshikawa.** 1988. Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. J. Bacteriol. **170:**2480–2484.
37. **Simons, K., and S. D. Fuller.** 1985. Cell surface polarity in epithelia. Annu. Rev. Cell Biol. **1:**243–288.
38. **Staunton, D. E., V. J. Merluzzi, and T. A. Springer.** 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell **56:**849–853.
39. **Takeichi, M.** 1987. Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. Trends Genet. **3:**213–215.
40. **Takeuchi, A.** 1967. Electron microscope studies of experimental Salmonella infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Pathol. **50:**109–136.
41. **Takeuchi, A., H. Sprinz, E. H. LaBrec, and S. B. Formal.** 1965. Experimental bacillary dysentery: an electron microscopic study of the response of the intestinal mucosa to bacterial invasion. Am. J. Pathol. **47:**1011–1044.
42. **Vasselon, T., J. Mounier, M. C. Prevost, R. Hellio, and P. J. Sansonetti.** 1991. Stress fiber-based movement of *Shigella flexneri* within cells. Infect. Immun. **59:**1723–1732.
43. **Vasselon, T., et al.** Submitted for publication.
44. **Volberg, T., B. Geiger, J. Kartenbeck, and W. W. Franke.** 1986. Changes in membrane-microfilament interaction in intercellular *adherens* junctions upon removal of extracellular Ca^{++} ions. J. Cell Biol. **102:**1832–1842.
45. **Wassef, J. S., D. F. Keren, and J. L. Mailloux.** 1989. Role of M cells in initial bacterial uptake and in ulcer formation in the rabbit intestinal loop model in shigellosis. Infect. Immun. **57:** 858–863.